STUDIES ON AMINO-ACIDS AND RELATED COMPOUNDS. PART VI. ELECTROLYTIC OXIDATION OF IMIDAZOLYL-PROPIONIC ACID.

By Yoshitaro TAKAYAMA.

Received April 13th, 1933. Published July 28th, 1933.

In the previous communications,[1] it was stated that pyrrolidone-carboxylic acid was easily oxidised at anode and yielded considerable amount of succinimide. This fact shows that this heterocyclic nucleus is particularly unstable towards anodic oxidation. Some amino-acids possess heterocyclic nuclei, which may undergo more or less individual change by the anodic oxidation. In the present paper, the study on imidazolyl-propionic acid is to be reported. Before trying some experiments on histidine, the above acid was selected, because it has similar structure as histidine, and differs only at one point, the lack of α-amino-group. It seems to be more plain in this case than in the case of histidine itself to investigate the mechanism of oxidation of imidazol nucleus.

As to the oxidation of imidazol derivatives, two reports are cited in the literatures. Wyss[2] oxidised imidazol with chromic acid and potassium permanganate. In the former case, no definite compound was isolated, while in the latter, formic acid was obtained. On the other hand Radziszewski[3] isolated oxamide and oxamic acid (?) when he oxidised imidazol with 3 per cent. solution of hydrogen peroxide.

Little definite information has been given relating to the electrolytic oxidation of heterocyclic compounds. Fr. Fichter and his collaborator[4] have electrolytically oxidised several compounds having pyrazolone nucleus (antipyrine and its allies) and obtained products which were not the same as those obtained by pure chemical methods. But in none of these cases the rupture of pyrazolone nucleus was found to occur.

Imidazolyl-propionic acid was oxidised under the same conditions as in the previous experiments. The temperature was kept at 30°C. by immersing the cell in the thermostat. The electrolysate assumed yellowish tint as the electrolysis proceeded, but the formation of melanin-like substances was not observed. The amount of electrity applied was about 6F./mol.

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(1) Y. Takayama, this Bulletin, **8** (1933), 125, 137.
(2), (3) Br. Radziszewski, *Ber.*, **17** (1884), 1289.
(4) Fr. Fichter and H. D. Montmollin, Helv. *chim. Acta*, **5** (1922), 256.

Nitrogen Distribution in the Electrolysate.

NH_3-N (MgO)	65.8 %
NH_3-N (NaOH)	12.3 %

From this result the presence of amide type compounds can be presumed. The electrolysate was thoroughly extracted with ether. Parts soluble (a) and insoluble (b) in ether were treated separately.

Part Soluble in Ether (a). When ether was expelled there remained a certain amount of crystalline residue, which was separated from a small bulk of the mother liquor. After recrystallisation from water, it melted at 184°C. and was found to be succinic acid by elementary analysis. The further crop* of this acid was obtained from (b) by other treatments. The joined yield of succinic acid amounted to 30.4% of imidazolyl-propionic acid used.

The mother liquor of succinic acid showed fuchsin reaction and silver mirror reaction. *p*-Nitrophenylhydrazone was prepared from it. The hydrazone, after recrystallisation from alcohol, melted at 172–174°C. The aldehyde was found to be succinic acid semialdehyde by the analysis of this hydrazone.

Part Insoluble in Ether (b). An excess of barium hydroxide was added to it and the solution was distilled under reduced pressure until the final distillate showed no reaction of ammonia. The distillate was received in hydrochloric acid, from which ammonia was obtained. It was confirmed by the analysis of its platinic double chloride. The residue was freed from barium, and concentrated under reduced pressure to a small bulk (c), then the estimation of urea was carried out by the following methods.

(1) Xanthydrol Method. The definite volume of the solution (c) was taken and the methyl alcohol solution of xanthydrol (Kahlbaum) $O<\begin{matrix}C_6H_4\\C_6H_4\end{matrix}>CHOH$ was added to it. Urea could be quantitatively precipitated as dixanthyl urea.

(2) Urease Method. Urease was added to the same definite volume. The amount of urea was estimated by the titration of ammonia formed by the enzymatic action of urease.

The results obtained from (1) and (2) fairly coincided with each other and the yield of urea was 1.3% of imidazolyl-propionic acid used. The result of nitrogen distribution shows that the total amount of the

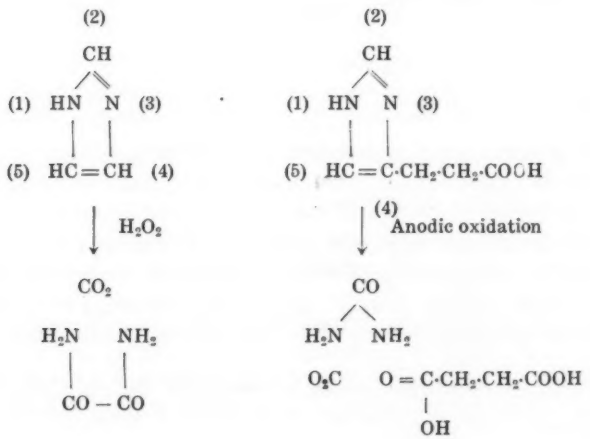
* Though sufficient quantity of ether was used, the extraction of succinic acid was not complete owing to its small solubility in that solvent.

amide compounds amounted to 12.5%. The discrepancy of the results obtained by different determinations may be ascribed to the presence of other compounds of amide type, such as oxamide or oxamic acid. But the project to obatin them from the solution was not successful. The difference is probably caused by the hydrolysis of urea during many treatments for its isolation.

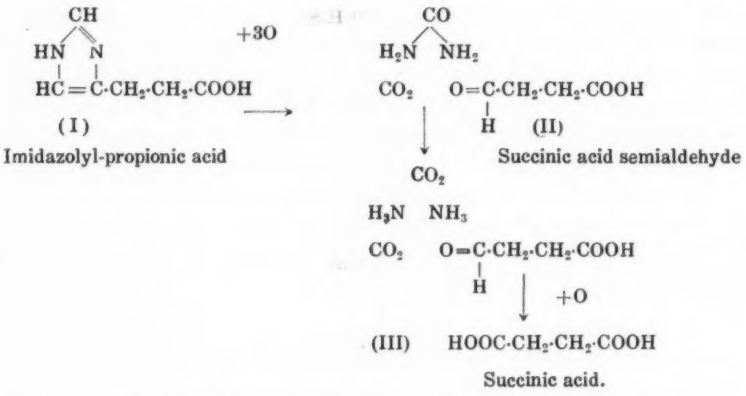
Remaining part of the solution (c) was concentrated to a syrup under reduced pressure and was extracted with hot isobutyl alcohol, and the crystals were obtained from the extract. It was found to be unchanged imidazolyl-propionic acid.

Mechanism of Oxidation of Imidazol Compounds.

As already mentioned[3] oxamide and oxamic acid were isolated by Radziszewski by the oxidation of imidazol. In this case it must be considered that the oxidation of imidazol took place at its carbon (2).



On the contrary, in the above anodic oxidation large amount of succinic acid together with little urea was isolated. This time the oxidation proceeded through another course, imidazol nucleus being attacked at carbon(5). It may be concluded that imidazol nucleus, on oxidation, behaves differently according to the kinds of oxidising agents (and probably to the presence or absence of the side chain). The mechanism of the anodic oxidation of imidazolyl-propionic acid may be represented as follows.



Succinic acid semialdehyde might as well be considered as the product of the secondary cathodic reduction of succinic acid, but the course of formation above cited was justified by the experiments reported in the previous communication.[5]

Experimental Part.

Imidazolyl-propionic acid[6]

Anal.: Subst. = 0.2347 gr. NH_3 = 33.00 c.c. of N./10-H_2SO_4,
Found: H = 19.70%.
Calc. for $C_6H_8O_2N_2$: N = 19.99%.

4.2 Gr. of imidazolyl-propionic acid (ca. 30 millimol) were dissolved in 100 c.c. of 2N.-sulphuric acid and electrolysed under the following conditions. Cell: without diaphragm. C.D. = 2 amp/dm². Electrodes (3×7 cm.): lead peroxide—lead. Electric quantity applied: 6.58 F/mol.

Distribution of Nitrogen. The electrolysate was filtered and made up to 125 c.c. 10 c.c. of the solution were taken for the following determinations,

(1) An excess of magnesia was added and distilled under reduced pressure.

(5) Y. Takayama, this Bulletin, **8** (1933), 125: Succinic acid was electrolytically oxidised under the same conditions as above. The electrolysate did not show characteristic reaction of aldehyde.

(6) S. Akabori, *J. Chem. Soc. Japan*, **52** (1931), 849. The sample of imidazolyl-propionic acid was kindly offered by Dr. Akabori and Mr. Numano, to whom the author is very grateful.

NH_3 = 31.68 c.c. of N./10-H_2SO_4	N = 44.2 mg.
total-N in 10 c.c. (calc.)	N = 67.2 mg.
NH_3-N(MgO)/total-N	65.8%.

(2) 30% sodium hydroxide solution was added to the residue and distilled again.

NH_3 = 5.82 c.c. of N./10-H_2SO_4	N = 8.28 mg.
total-N in 10 c.c. (calc.)	N = 67.2 mg.
NH_3-N(NaOH)/total-N	12.3%.

It was previously ascertained that imidazolyl-propionic acid did not yield ammonia when it was boiled with concentrated sodium hydroxide.

Succinic Acid. The oxidised solution was thoroughly extracted with ether. The colourless crystals were obtained from the extract. (0.576 gr.). After recrystallisation from water it melted at 184°C.

Anal.: Subst. = 0.0607; CO_2 = 0.0904; H_2O = 0.0275 gr.
Found: C = 40.61; H = 5.07%.
Calc. for $C_4H_6O_4$ (succinic acid): C = 40.63; H = 5.12%.

The residue of the extraction was evaporated to a syrup under reduced pressure and extracted with acetone. A crystalline residue (0.540 gr.) was obtained from the extract, it was found to be nothing else than succinic acid. The joined yield of succinic acid amounted to 1.116 gr. (9.45 millimol) representing 31.4% of imidazolyl-propionic acid used.

Succinic Acid Semialdehyde. The viscous mother liquor of succinic acid was kept over concentrated sulphuric acid under reduced pressure and crystallised succinic acid was filtered off. Following the description of Carrière,[7] to the filtered solution of *p*-nitrophenylhydrazine (0.5 gr.) in acetic acid (3 gr.) the aldehyde was added. Reddish yellow precipitate formed was collected and drained on porous plate. After repeated recrystallisation from alcohol, with addition of animal charcoal, it melted at 172–174°C.

The melting points of *p*-nitrophenylhydrazone of succinic acid semialdehyde given by many authors are as follows.

Alefeld[8]	175°C.	1909
Langheld[9]	174°C.	1909
Harries[10]	177°C.	1912
Dakin[11]	185–187°C. (when rapidly heated)	1917
Carrière	180–181°C.	1922

(7) Carrière, *Ann. chim.*, [9] **17** (1922), 75.
(8) Alefeld, *Ber.*, **42** (1909), 165, 1426.
(9) Langheld, *Ber.*, **42** (1909), 2371.
(10) Harries, *Ber.*, **45** (1912), 2585.
(11) Dakin, *Biochem. J.*, **11** (1917), 84.

The value obtained by the author (172–174°C.) was lower than any of the above. But the sample was too minute to try further recrystallisation.

Anal.:[12] Subst. = 7.264 mg.; NH_3 = 6.24 c.c. of N./70-H_2SO_4.
Found: N = 17.18%.
Calc. for $C_{10}H_{11}O_4N_3$: N = 17.7%.

Ammonia and Urea. An excess of barium hydroxide was added to the solution (b) and it was distilled under reduced pressure. The distillate was received in hydrochloric acid and the volatile base was collected as hydrochloride. It was found to be ammonia by the analysis of its platinic double chloride.

Anal.: Subst. = 0.1712; Pt = 0.0746 gr. Found: Pt = 43.6%.
Calc. for $(NH_4)_2PtCl_6$: Pt = 43.9%.

The excess of barium was removed exactly and the solution was concentrated to 50 c.c. under reduced pressure. Urea was estimated as follows.

(1) Xanthydrol Method.[13] 5 c.c. out of 50 c.c. (corresponding to 8/100 part of the total volume) were taken and poured into three parts of acetic acid, to which 3 c.c. of 7% methyl alcoholic solution of xanthydrol (Kahlbaum) were added. After the solution was allowed to stand overnight, the flocculent precipitate was collected on a filter. The dixanthyl urea obtained weighed 12.0 mg. corresponding to 1.71 mg. urea.

Anal.: Subst. = 8.169 mg.; NH_3 = 2.60 c.c. of N./70-H_2SO_4
Found: N = 6.36%.
Calc. for $C_{27}H_{20}O_3N_2$: N = 6.66%.

(2) Urease Method.[14] The same volume as in the above method was taken and it was neutralised. A buffer solution of phosphates and 0.1 gr. of urease (Squibb) were added to it. After the solution was kept at 40–50°C. for 15 minutes, an excess of sodium carbonate was added and the rapid current of air was passed through the solution, transferring the formed ammonia into N./70-H_2SO_4 (method of Folin). The same treatment was repeated without adding urease.

(a)	Urease added: N./70-H_2SO_4 6.74 c.c.	N = 1.348 mg.
(b)	Without urease: N./70-H_2SO_4 2.29 c.c.	N = 0.458 mg.
	diff.	N = 0.890 mg.
	calc. for urea	1.91 mg.

(12) As this compound contains nitro and hydrazo groups, it must be previously reduced. Zn and alcohol were chosen as reducing agent. See Hans Meyer's Methodik (1931) p. 426 and Weyl's Methoden, I (1921) p. 52.
(13) Fosse, *Ann. chim.*, (9), **6** (1916), 67.
(14) See Hoppe-Seyler, Thierfelder, „Physiologisch- und pathologisch-chemische Analyse" (1924), p. 702.

The urease used was ascertained beforehand not to contain an appreciable amount of ammonia. Therefore, ammonia formed in (b) may have been produced by the hydrolysis of urea during the concentration of the solution.

Imidazolyl-propionic Acid. The remaining part of the solution was concentrated to a syrup under reduced pressure and was extracted with hot isobutyl alcohol. The solvent was expelled and the residue was redissolved in a small bulk of water. A large amount of acetone was added to it. Colourless crystals were deposited on the wall of the beaker, which melted at 201–202°C. It was found to be imidazolyl-propionic acid by the determination of nitrogen and mixed melting point test.

Anal.: Subst. = 7.883 mg.; NH_3 = 7.90 c.c. of N./70 H_2SO_4.
Found: N = 20.04%.
Calc. for $C_6H_8O_2N_2$: N = 19.99%.

In conclusion, the author wishes to express his sincere thanks to Professor K. Matsubara for his kind encouragement and invaluable advices given to this study, and also to Mr. H. Ôeda for his untired assistance.

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FREEZING POINT CURVES OF BINARY MIXTURES: $TiCl_4$-$SiCl_4$, $TiCl_4$-CCl_4, $TiCl_4$-$SnCl_4$ AND $TiCl_4$-$SbCl_5$.

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Received June 10th, 1933. Published July 28th, 1933.

Synopsis.

Freezing point curves of binary mixtures, $TiCl_4$-$SiCl_4$, $TiCl_4$-$SnCl_4$, $TiCl_4$-CCl_4, and $TiCl_4$-$SbCl_5$ have been determined by the methods of both total and differential thermal analyses. In the system $TiCl_4$-$SiCl_4$ an eutectic point lies on the position almost 100% of $SiCl_4$, and with the mixtures $TiCl_4$-CCl_4 and $TiCl_4$-$SbCl_5$ a simple eutectic system has been shown, while the mixture $TiCl_4$-$SnCl_4$ shows a simple solid solution.

Introduction.

Titanium tetrachloride can be prepared from titanium dioxide which is usually found in the iron sand. Consequently the investigation of

titanium tetrachloride has very profound interest with the industrial application of the iron sand. The present author has intended as a first attempt to investigate the freezing point curves of the binary mixtures of liquid chlorides containing titanium tetrachloride.

Experimental Method.

1. Sampling of the Chlorides avoiding those from Decomposition. It will be difficult to prepare mixtures of known composition, which is extremely smoky in the presence of wet atmosphere, such as, those chlorides (excepting carbon tetrachloride), by weighing them with a balance. There is also a method of measurement by which these can be distillated in a measuring apparatus which is kept in vacuum, but then this will be of greatly complicated in such a case as to determine the freezing point curve. Hence in the present experiment the volumes of these chlorides have been measured with burettes avoiding them contact with moisture, and calculated the composition of these mixtures from their density. All these chlorides were purified by the distillation before use. Stocked chlorides were kept in flasks each of which has a side tube as shown in Fig. 1 A, and when those were used, dried air were let in from the tube avoiding the contact of moisture with chlorides. When the chlorides were let into a burette from the stocked flask through a long mouthed funnel inserted into a tube *a* shown in Fig. 1, dry air was sent from the tube *b* letting it out from the funnel, avoiding the contact of moisture with the chlorides, and taking the lid *c* off to allow the chlorides into the burette. After the chlorides were let into the burette, the lid *c*, being replaced the funnel being removed and the tube *a* was closed, at the same time, sending of the dry air from *b* was ceased. *d* in Fig. 1 and *d'* in Fig. 2 are gum stoppers whose surfaces are previously attacked by the chloride vapour for a sufficiently long time to avoid chlorides from being contaminated. Two grooves are dug on the stop-cock as shown in the figure and mercury is poured in them to protect the chlorides from

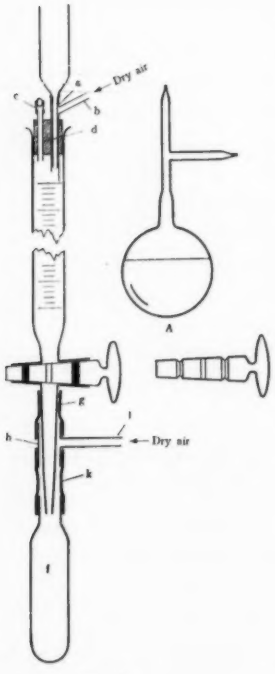

Fig. 1.

the moisture and to keep it movable. Although the boundary between the mercury and the chlorides became brownish black due to the contamination of the mercury during the experiment, the phenomenon was simply confined in the boundary; hence, the chlorides in the burette were able to keep their original purity. The purity was further assured by discording the first small quantity of chlorides into *f*, before measuring the chlorides out from the burette into the apparatus. *g* and *k* are gum tubes and *h* is made of glass. The measuring of the chlorides into the apparatus was consisted of lowering *g*, *h* and *k* downwards, passing the dry air very slowly from tube *l*, connecting the side tube *m* in Fig 2 with rubber tube *k*, at the same time *f* is removed and while opening the lid *n* allowing the air coming out, the desired quantity of the chlorides was measured into the apparatus. When the measurement was completed, *m* is removed from *k*, letting the dry air pass through from *n*, and *m* is quickly covered up, and finally the air supply from *n* was discontinued. *f* is re-set into *k*, at the same time letting the dry air cease to come from *l*. When carbon tetrachloride was used, an usual burette was employed, as it does not fume in the wet air. Since the data available in the literature relating to the density of these chlorides are very meagre, the density of these chlorides was calculated by the interpolation of several values in the literature. The references on the value adopted are as follows; the density of titanium tetrachloride from T. Sagawa,[1] a member of our institute, of silicon tetrachloride from T. E. Thorpe,[2] O. Ruff, and C. Albert,[3] of carbon tetrachloride from T. E. Thorpe[4] and Joseph,[5] of stannic chloride from Young and Fortey,[6] of antimony tetrachloride from A. Haagen,[7] O. Ruff, and W. Plato.[8]

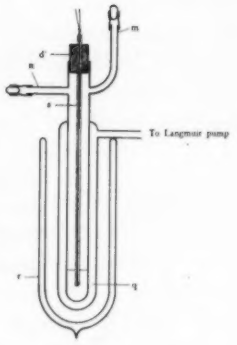

Fig. 2.

2. Thermal Analyses. The cooling velocity is regulated by the pressure at the space *q* in Fig. 2 to which Langmuir pump is connected. *r* is a Dewar vessel containing liquid nitrogen or mixture of solid carbon

(1) T. Sagawa, *Kinzoku no Kenkyū*, **9** (1932), 189.
(2) T. E. Thorpe, *J. Chem. Soc.*, **37** (1880), 327.
(3) O. Ruff, C. Albert, *Ber.*, **38** (1905), 2222.
(4) T. E. Thorpe, *Trans. Chem. Soc.*, **37** (1880), 141.
(5) Joseph, *Trans. Chem. Soc.*, **107** (1915), 1.
(6) Young & Fortey, *J. Chem. Soc.*, **81** (1902), 783.
(7) A. Haagen, *Pogg. Ann.*, **131** (1867), 122.
(8) O. Ruff & W. Plato, *Ber.*, **37** (1904), 679.

dioxide and ether. *s* is a protecting glass tube in which one junction of the copper-constantan thermocouple is inserted, and the other junction is inserted in the melting ice. The temperature was read by a pyrometer. The copper-constantan thermocouple was previously calibrated by the melting points of pure chlorobenzene, chloroform, ethyl acetate, toluene, ethyl ether, and mercury, and also by the boiling point of liquid nitrogen. On the differential thermal analysis the mixture of the desired composition was prepared firstly in the flask shown in Fig. 3 with the burette shown in Fig. 1, and then about 0.05 c.c. of the mixture was introduced into the glass tube whose diameter is 3 mm. shown in Fig. 4. The purpose of using such a small quantity of chloride was to make the uniform temperature at the extremity of the thermocouple. In Fig. 4 *t* is a copper block, 3 cm. in diameter and 4 cm. in length, into which two glass tubes are inserted; the one contains chloride, and the other water. In the tube containing chloride two junctions, each end of the temperature measurement thermocouple and of the differential thermal analysis, are inserted. These two junctions are inserted respectively into two capillaries which are placed symmetrically about the inner wall of the tube containing chloride. Two wires of the junction are insulated with the oxide coating on the copper wire made by the Bunsen burner. When the differential thermal analysis was carried on the copper block was previously dipped in liquid nitrogen, solidifying both the chloride and the neutral matter (water), and they were then taken out in the air, and finally the heating curves were drawn from the readings of a galvanometer. The velocity of the heating was about 0.4° per minute.

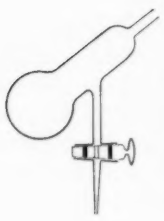

Fig. 3.

Fig. 4.

In the case of the total thermal analysis the solidus points on both sides of the eutectic point in the system $TiCl_4$-CCl_4 were indicated slightly lower temperature on the cooling curve and somewhat higher temperature on the heating curves. The similar phenomena were observed on the antimony pentachloride rich-side in the system $TiCl_4$-$SbCl_5$; consequently in both diagrams the mean values of these temperatures were adopted. Curves of the thermal analyses were shown in Figs. 9 and 10, and the results were tabulated and illustrated diagramatically in next paragraph.

Results of Experiment.

(1) $TiCl_4$-$SiCl_4$. When the cooling curve of the total thermal analysis was taken in this system, the eutectic flat did not appears until the

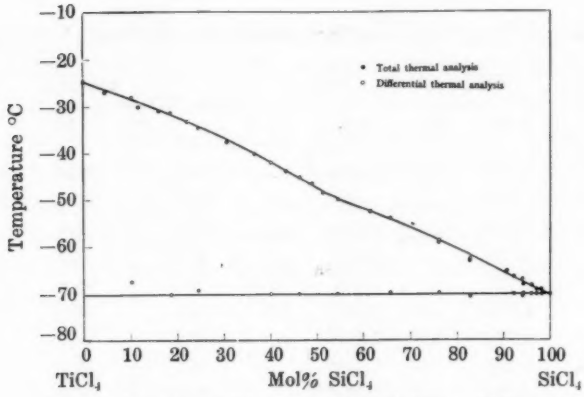

Fig. 5. Freezing point curve of the mixture: $TiCl_4$—$SiCl_4$.

1. $TiCl_4$-$SiCl_4$

Table 1. Total Thermal Analysis (Cooling Curves)

No.	Temp.	$TiCl_4$		$SiCl_4$		Mol% $SiCl_4$	Liquidus point —°C	Eutectic point —°C
		s.g.	c.c.	s.g.	c.c.			
1	15.4	1.7354	5.0	1.4927	0.0	0.0	24.8	—
2	15.5	1.7352	,,	1.4926	0.25	4.6	27.0	—
3	15.3	1.7356	,,	1.4928	0.7	11.9	30.3	—
4	15.5	1.7353	,,	1.4926	1.0	16.2	31.0	—
5	15.8	1.7346	,,	1.4924	1.5	22.4	33.4	—
6	14.2	1.7376	,,	1.4937	2.3	30.6	37.6	—
7	14.5	1.7370	,,	1.4935	3.0	36.6	40.4	—
8	15.1	1.7360	,,	1.4930	4.0	43.5	43 9	—
9	13.2	1.7393	,,	1.4946	5.0	49.0	46.7	—
10	12.1	1.7410	,,	1.4755	5.5	51.3	48.7	—
11	12.2	1.7408	3.0	1.4954	5.0	61.5	52.6	—
12	12 5	1.7403	2.0	1.4952	,,	70.6	55.2	—
13	13.5	1.7386	1.5	1.4943	,,	76.2	59.3	—
14	16.5	1.7336	1.0	1.4918	,,	82.8	63.3	—
15	15.4	1.7352	0.5	1.4927	,,	90.6	65.3	—
16	14.8	1.7364	0.4	1.4932	,,	92.3	66.8	70.3
17	15.9	1.7346	0.3	1.4923	,,	94.1	68.3	70.3
18	14.1	1.7376	0.2	1.4938	,,	96.0	68.3	70.3
19	13 2	1.7391	0.15	1.4946	,,	97.0	69.3	70.3
20	15.1	1.7359	0.1	1.4930	,,	98.0	69.3	70.3
21	15.1	1.7359	0.0	1.4930	,,	100.0	70.4	—

Table 2. Differential Thermal Analysis (Heating Curves)

No.	Temp.	$TiCl_4$		$SiCl_4$		Mol% $SiCl_4$	Liquidus point —°C	Eutectic point —°C
		s.g.	c.c.	s.g.	c c.			
22	12.5	1.7403	5.0	1.4952	0.6	10.3	28.0	67.3
23	13.0	1.7394	,,	1.4948	1.2	18.7	31.4	70.3
24	13.4	1.7388	,,	1.4944	1.7	24.6	34.6	69.3
25	13.5	1.7386	,,	1.4943	3.5	40.2	42.9	70.3
26	12.5	1.7303	,,	1.4952	4.5	46.3	45.3	70.3
27	12.8	1.7398	4.0	1.4949	5.0	54.5	50.4	70.3
28	13.2	1.7391	2.5	1.4946	,,	65.8	54.2	70.3
29	13.4	1.7388	1.5	1.4944	,,	76.2	58.9	70.3
30	13.8	1.7381	1.0	1.4941	,,	82.8	60.3	70.9
31	13.5	1.7386	0.3	1.4943	,,	94.1	66.6	70.9

mixture contained a quite large quantity of $SiCl_4$, say 90.6 Mol % $SiCl_4$. In the case of the differential thermal analysis the eutectic points were observed until the mixtures contained as small quantity of $SiCl_4$ as 10.3 Mol %. In both analyses this eutectic point was perfectly equal to the freezing point of pure $SiCl_4$. Liquidus curve descends, as shown in Fig. 5, gradually from 100% $TiCl_4$ to 100% $SiCl_4$. In another words in this system when a mixture freezes, $TiCl_4$ crystallizes out firstly, and until all the $TiCl_4$ freezes out, the crystallization of $SiCl_4$ does not begin.

(2) **$TiCl_4$-CCl_4.** In this system both the liquidus and the eutectic point appeared distinctly, as shown in Fig. 6, even in the case of the

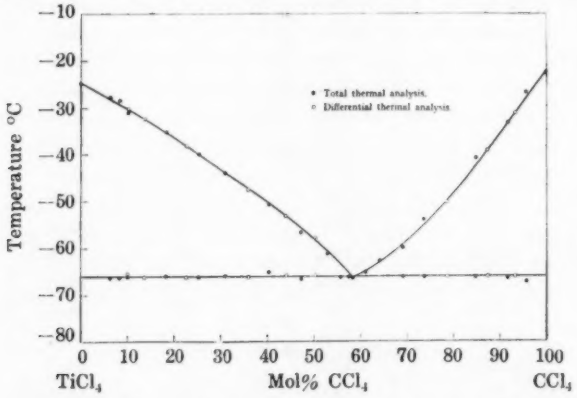

Fig. 6. Freezing point curve of the mixture: $TiCl_4$—CCl_4.

total thermal analysis; but the eutectic point found to be somewhat higher temperature in the heating curves than that of the cooling. This difference grew greater and greater as the composition of the mixture remote from the eutectic point, as shown in Table 3; hence in the figure mean values have been plotted. Perhaps these abnormalities can be removed if small quantity of the sample is used. In the case of the differential thermal analysis, therefore, small quantities of the samples were employed as described before, and almost definite eutectic temperature was observed.

2. $TiCl_4$-CCl_4

Table 3. Total Thermal Analysis (Cooling Curves)

No.	Temp.	$TiCl_4$		CCl_4		Mol % CCl_4	Liquidus point —°C	Eutectic point —°C		
		s.g.	c.c.	s.g.	c.c.			Cooling	Heating	Mean
1	16.0	1.7344	5.0	1.5817	0.0	0.0	24.8	—	—	—
2	16.1	1.7341	,,	1.5816	0.3	6.3	27.6	75.2	57.3	66.3
3	16.5	1.7336	,,	1.5813	0.4	8.3	28.5	75.2	57.3	66.3
4	16.8	1.7331	,,	1.5810	0.5	10.1	31.0	74.0	58.0	66.0
5	17.9	1.7311	,,	1.5801	1.0	18.4	35.4	74.0	58.0	66.0
6	18.2	1.7308	,,	1.5799	1.5	25.3	40.0	73.5	59.0	66.1
7	17.6	1.7318	,,	1.5804	2.0	31.0	44.3	73.0	59.3	65.8
8	18.1	1.7309	,,	1.5800	3.0	40.3	50.7	70.2	59.8	65.0
9	19.0	1.7294	,,	1.5793	4.0	47.4	56.7	68.8	64.3	66.6
10	20.8	1.7264	,,	1.5778	5.0	53.0	61.1	66.8	—	—
11	21.3	1.7256	,,	1.5774	5.6	55.8	—	66.3	—	—
12	21.8	1.7247	,,	1.5770	6.5	57.5	—	66.3	—	—
13	23.9	1.7211	4.0	1.5753	5.0	58.5	—	66.5	—	—
14	22.8	1.7230	5.0	1.5762	7.0	61.2	65.3	67.8	—	—
15	23.0	1.7227	5.0	1.5960	8.0	64.4	62.8	67.8	—	—
16	24.5	1.7202	2.5	1.5748	5.0	69.3	60.3	69.3	63.2	66.3
17	24.2	1.7207	2.0	1.5750	,,	73.8	54.2	70.2	62.3	66.3
18	25.9	1.7178	1.0	1.5736	,,	85.0	41.3	72.0	60.6	66.3
19	25.2	1.7190	0.5	1.5742	,,	91.9	33.4	73.0	60.0	66.5
20	26.5	1.7168	0.25	1.5732	,,	95.8	26.9	75.2	59.3	67.3
21	26.4	1.7170	0.0	1.5733	,,	100.0	22.9	—	—	—

Table 4. Differential Thermal Analysis (Heating Curves)

No.	Temp.	$TiCl_4$		CCl_4		Mol% CCl_4	Liquidus point —°C	Eutectic point —°C
		s.g.	c.c.	s.g.	c.c.			
22	11.2	1.7424	5.0	1.5856	0.5	10.1	30.4	65.7
23	11.5	1.7420	,,	1.5854	0.7	13.6	32.4	66.3
24	12.5	1.7403	,,	1.5845	1.3	22.6	38.2	66.3
25	10.5	1.7436	,,	1.5862	2.5	35.9	47.6	66.1
26	12.3	1.7406	,,	1.5847	3.5	44.0	53.2	65.9
27	12.5	1.7403	,,	1.5846	4.5	50.3	58.1	65.9
28	12.8	1.7398	1.5	1.5843	5.0	78.9	50.2	66.1
29	12.7	1.7400	0.8	1.5844	,,	87.5	39.4	66.2
30	12.7	1.7400	0.4	1.5844	,,	93.4	31.4	66.1

(3) **$TiCl_4$-$SnCl_4$.** In this system, as shown by the cooling curves in Fig. 12, while the primary crystallization points were observed distinctly, the eutectic flat did not appear at all. From this fact it is to be assumed that this system is to form the solid solution; but in spite of this prospect, the decision as to whether this system would form the solid solution or not seemed inadequate merely by the total thermal analysis. Only when the differential thermal analysis was taken it was able to decide that all the mixtures in this system, whatever was the composition, would form the solid solution. In Fig. 7 liquidus points from cooling curves of the total thermal analysis and the solidus points from heating curves of the differential thermal analysis were plotted.

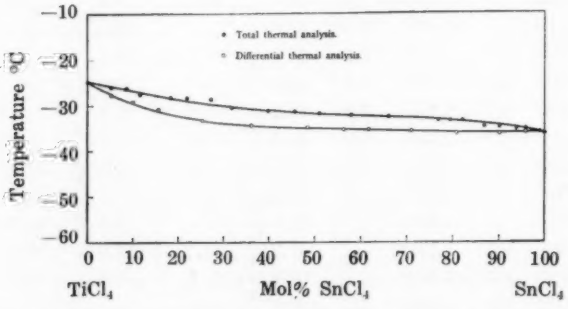

Fig. 7. Freezing point curve of the mixture: $TiCl_4$—$SnCl_4$.

3. $TiCl_4$-$SnCl_4$

Table 5. Total Thermal Analysis (Cooling Curves)

No.	Temp.	$TiCl_4$		$SnSl_4$		Mol% $SnCl_4$	Liquidus point —°C
		s.g.	c.c.	s.g.	c.c.		
1	26.7	1.7165	5.0	2.2088	0	0.0	24.8
2	26.8	1.7164	,,	2.2085	0.3	5.3	26.0
3	26.6	1.7167	,,	2.2090	0.5	8.6	27.1
4	27.1	1.7158	,,	2.2078	0.7	11.6	27.5
5	26.5	1.7168	,,	2.2093	1.2	18.4	28.3
6	26.4	1.7170	,,	2.2096	1.5	22.0	28.4
7	25.5	1.7185	,,	2.2119	2.0	27.3	28.6
8	25.4	1.7187	,,	2.2121	2.5	31.9	30.5
9	25.8	1.7180	,,	2.2111	3.5	39.6	31.2
10	25.7	1.7181	,,	2.2114	4.5	45.8	31.3
11	25.1	1.7192	,,	2.2129	5.5	50.8	31.6
12	24.2	1.7208	3.4	2.2152	5.0	58.0	32.2
13	23.8	1.7214	2.4	2.2163	,,	66.1	32.5
14	23.2	1.7240	1.4	2.2224	,,	77.0	33.4
15	22.4	1.7237	1.2	2.2199	,,	79.6	33.4
16	22.0	1.7244	1.0	2.2210	,,	82.4	34.4
17	20.0	1.7278	0.7	2.2262	,,	87.0	34.7
18	18.0	1.7311	0.5	2.2315	,,	90.4	34.8
19	19.5	1.7286	0.3	2,2275	,,	94.0	35.2
20	19.5	1.7286	0.2	2.2275	,,	95.9	35.4
21	19.3	1.7889	0.05	2.2280	,,	98.9	35.8
22	19.4	1.7287	0.0	2.2278	,,	100.0	36.2

Table 6. Differential Thermal Analysis (Heating Curves)

No.	Temp.	$TiCl_4$		$SnCl_4$		Mol% $SnCl_4$	Solidus point —°C
		s.g.	c.c.	s.g.	c.c.		
23	11.0	1.7428	5.0	2.2497	0.3	5.3	27.9
24	11.2	1.7424	,,	2.2492	0.6	10.1	29.4
25	12.5	1.7403	,,	2.2458	1.0	15.8	30.8
26	13.0	1.7394	,,	2.2445	1.8	25.3	33.4
27	13.5	1.7386	,,	2.2432	3.0	36.1	34.4
28	12.5	1.7403	,,	2.2458	5.0	48.5	34.8
29	12.4	1.7404	3.65	2.2461	5.0	56.3	35.4
30	13.4	1.7388	2.9	2.2434	,,	61.8	35.4
31	12.8	1.7398	1.9	2.2450	,,	71.2	35.6
32	13.0	1.7394	1.1	2.2445	,,	81.0	36.1
33	12.0	1.7411	0.5	2.2472	,,	90.4	36.4
34	14.2	1.7374	0.2	2.2414	,,	96.0	35.9

(4) **$TiCl_4$-$SnCl_5$.** From the results of the total and the differential thermal analyses, it has been found that the eutectic could be observed regularly in this system. But when cooling and heating curves of total thermal analysis were drawn the eutectic temperature varied only on the $SbCl_5$ rich side.

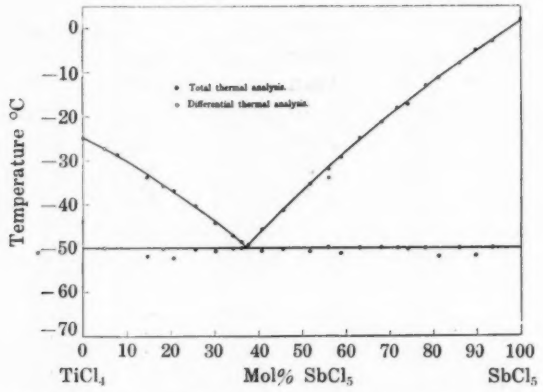

Fig. 8. Freezing point curve of the mixture: $TiCl_4 - SbCl_5$.

4. $TiCl_4$-$SbCl_5$

Table 7. Total Thermal Analysis (Cooling Curves)

No.	Temp.	$TiCl_4$		$SbCl_5$		Mol% $SbCl_5$	Liquidus point −°C	Eutectic point −°C		
		s.g.	c.c.	s.g.	c.c.			Cooling	Heating	Mean
1	26.1	1.7176	5.0	2.3325	0.0	0.0	24.8	—	—	—
2	26.0	1.7177	,,	2.3327	0.5	7.9	28.7	—	—	—
3	26.2	1.7174	,,	2.3322	1.0	14.7	33.8	51.6	—	—
4	26.1	1.7175	,,	2.3324	1.5	20.5	36.8	52.2	—	—
5	25.8	1.7180	,,	2.3331	2.0	25.6	40.4	50 2	—	—
6	25.5	1.7185	,,	2.3338	2.5	30.1	44.4	50.6	—	—
7	25.6	1.7184	,,	2.3336	3.0	34.1	47.2	50.2	—	—
8	25.8	1.7180	,,	2.3331	3.3	36.3	48.7	50.0	—	—
9	25.7	1.7182	,,	2.3335	3.5	37.6	—	49.2	—	—
10	26.0	1.7177	,,	2.3327	4.0	40.8	45.7	50.7	—	—
11	25.6	1.7184	,,	2.3336	5.0	46.3	41.4	50.2	—	—
12	25.8	1.7180	4.0	2.3331	5.0	51.9	35.4	50.7	—	—
13	25.6	1.7184	3.0	2.3336	,,	59.0	29.4	51.2	—	—
14	25.7	1.7182	2.0	2.3334	,,	68.3	21.3	53.2	46.2	49.7
15	25.8	1.7180	1.5	2.3331	,,	74.2	17.3	52.7	47.7	50.2
16	25.8	1.7180	1.0	2.3331	,,	81.2	11.3	57.8	45.2	51.9
17	25.5	1.7185	0.5	2.3338	,,	89.6	5.0	59.3	43.8	51.7
18	25.4	1.7187	0.0	2.3340	,,	100.0	+ 2.0	—	—	—

Table 8. Differential Thermal Analysis (Heating Curves)

No.	Temp.	$TiCl_4$		$SbCl_5$		Mol% $SbCl_5$	Liquidus point —°C	Eutectic point —°C
		s.g.	c.c.	s.g.	c.c.			
19	12.5	1.7403	5.0	2.3627	0.3	4.9	27.3	50.0
20	12.3	1.7406	,,	2.3631	1.3	18.3	35.8	50.2
21	12.4	1.7404	3.5	2.3629	5.0	56.0	34.0	49.8
22	12.3	1.7406	2.5	2.3631	,,	63.3	25.1	50.2
23	12.3	1.7406	1.7	2.3631	,,	71.7	18.1	50.0
24	12.4	1.7404	1.2	2.3629	,,	78.2	13.1	50.0
25	12.5	1.7403	0.7	2.3627	,,	86.0	8.0	50.2
26	12.5	1.7403	0.3	2.3627	,,	93.5	3.0	50.0

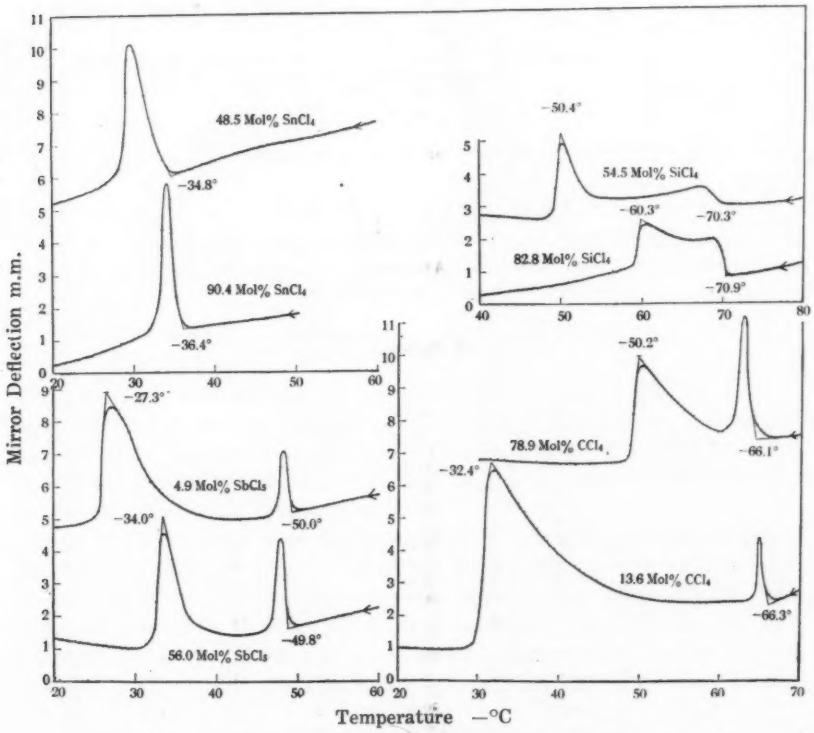

Fig. 9. Differential Thermal Analysis (Heating Curves).

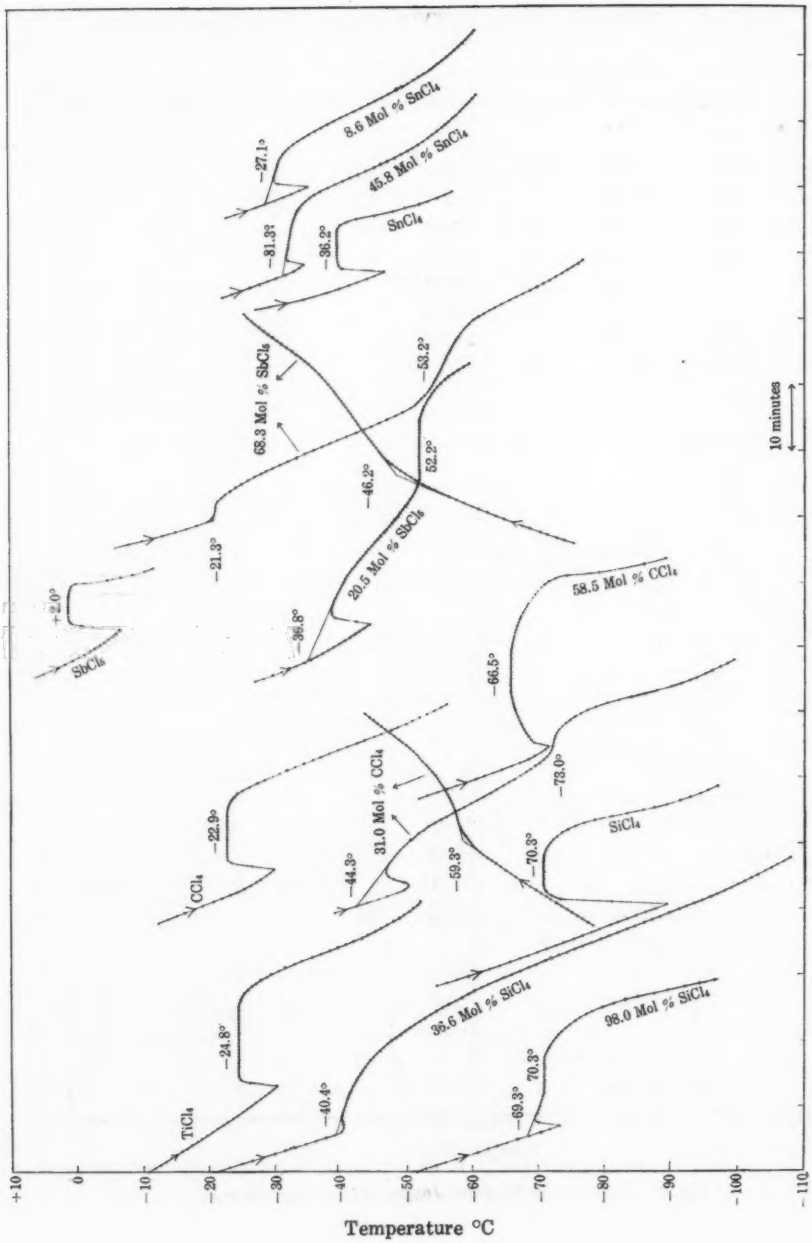

Fig. 10. Total Thermal Analysis.

As almost all of the cooling curves shows the supercooling of samples, primary crystallization points were decided by the back extrapolation method as shown in Fig. 10. In the case of the differential thermal analysis the points of intersection of the curves were taken as the required points.

In conclusion the present writer wishes to express his hearty thanks to Prof. Dr. K. Iwasé for his kind guidance, and thanks are also due to Mr. T. Okamura for his valuable advice.

Summary.

1. The apparatus was devised for the measurement of the liquids which smokes in the wet atmosphere without letting them smoke.

2. Using the apparatus described above the freezing point curves of the mixtures, $TiCl_4$-$SiCl_4$, $TiCl_4$-CCl_4, $TiCl_4$-$SnCl_4$, and $TiCl_4$-$SbCl_5$ were determined.

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GLUCO-SULFATASE VI.[1] ON NATURAL SUBSTRATES OF THE ENZYME.

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Received June 14th, 1933. Published July 28th, 1933.

In the preceding papers we have chiefly dealt with the properties, the purification of the enzyme and its distribution in molluscan phylum as well as in organs of the animal. It was, however, since the beginning of our research a problem what rôle does the enzyme, glucosulfatase, play in the physiology of those animals.

It is well known that the substrates of pheno-sulfatase naturally occur in the animal body. Couldn't we expect the corresponding substrate of glucosulfatase? Glucose-mono-sulphate, so far as we know, has not yet been found in nature. We have, however, observed during our experiments that the tissues of those animals seem to contain some organic sulphate and to liberate inorganic sulphate by the enzyme. Therefore,

(1) Gluco-sulfatase. Part I; III; V; this Bulletin, **6** (1931), 258; **8** (1933), 65; **8** (1933), 148. Part II; IV; *J. Chem. Soc. Japan*, **54** (1933), 59, 377.

it would be interesting and worth while to look for such a natural substrate in the body of those animals.

Of course, the merit of the enzyme would not be restricted merely to a single physiological function. It is known that there are some organic sulphates in fronds and in animal tissues, and these might be eaten and digested by those animals which contain gluco-sulfatase. Some kinds of gastropoda, such as *Dolium galea* or *Natica ampla*, are said to excrete sulphuric acid. It is very attractive to suppose some relation between those phenomena and the physiological rôle of our enzyme, but at present, it is merely a speculation. In the present paper, as a preliminary research, we have dealt with the distribution of ethereal sulphate in some forms of gastropoda.

The estimations of organic and inorganic sulphates were made on 5 kinds of animals. On *Viviparus japonicus* Martens (mud-snail) the body parts were roughly separated and the sulphates were estimated in each part. In muscular and organ parts the contents of organic sulphate were nearly equal; in embryo the content was markedly high, being 4-5 times of the content of muscular part. (Table I).

On other 4 species of marine origin the sulphates were determined not separately in parts, but the shell contents were boiled and crushed altogether in each case and their sulphates were determined. In all cases, the contents of organic sulphate (as S) were higher than that of *Viviparus* (9.8 mg.), ranging diversely from 10.4 up to 259 mg. per 100 gr. (see Table II). From the results it would be obvious that the order of activities of enzyme is about the same as that of contents of organic sulphate. The latter, it is worthy of special mention, is just the same as that of the relative weight of the shells, thus suggesting the intimate relation between shell-formation and organic sulphate as well as gluco-sulfatase (see Table III).

The extraction by sodium chloride solution was ineffective in all cases. At the best result (*Viviparus*, muscular part) 1/2.4 of organic sulphate was extracted and at the worst one only 1/6.5 was extracted, and yet it is probable that the organic sulphate in the extract may be due to the suspending proteinous matter. Contrary to chondroitin-sulphuric acid ester, therefore, the substance seems to be difficultly soluble in sodium chloride solution. Alcohol and acetone were much more inefficient for extraction.

Some insoluble parts containing organic sulphate were subjected to peptic digestions, but in many cases they went very slowly. But the embryo of *Viviparus* was dissolved by pepsin-HCl in 2 days and the solution showed the presence of a considerable amount of organic sulphate.

The mucus secreted from fresh *Charonia* by pouring chloroform into its shell showed very interesting properties; it is coagulated by acetone or by boiling, giving gelatinous coagulum and it contains much organic sulphate together with a large quantity of inorganic sulphate. The coagulum did not dissolve in peptic and tryptic mixtures and thus we could not obtain organic sulphate in soluble form. We found, however, the existence of soluble organic sulphate in the filtrate of heat-coagulum. This filtrate, when mixed with the enzyme obtained from the liver of *Charonia*, showed some increase of inorganic sulphate, and thus we could ascertain the hydrolysis of this soluble organic sulphate by gluco-sulfatase. Furthermore, it is clear from our experiment that the insoluble organic sulphate was also hydrolysed by the sulfatase. (58.2% of S of insoluble organic sulphate was hydrolysed during 2 weeks' digestion by the enzyme). From the above results we are convinced of the existence of substrate of gluco-sulfatase probably both in soluble and insoluble form in the bodies of some shell-fishes.

Experimental Details.

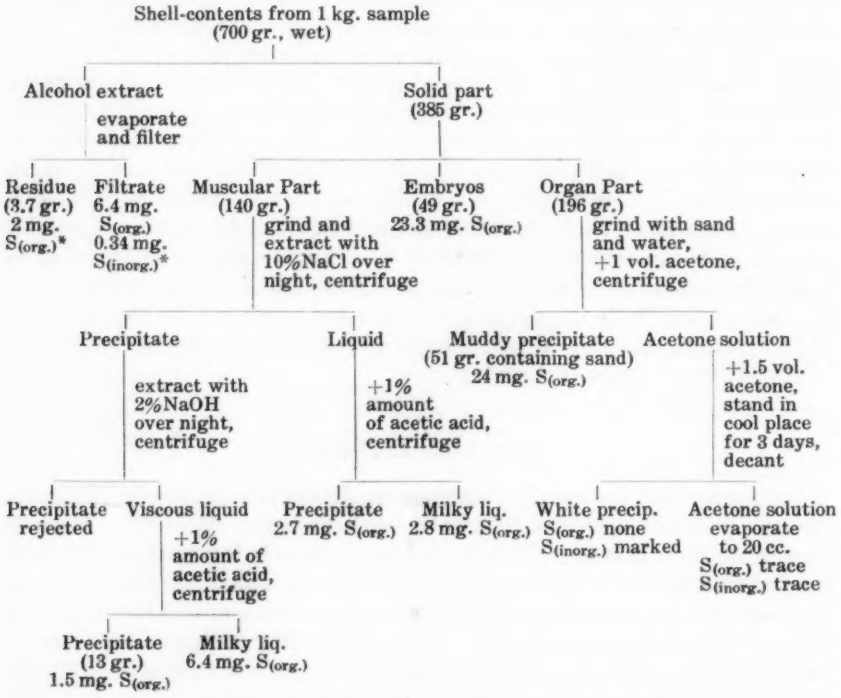
Organic and Inorganic Sulphates in *Viviparus japonicus*, Martens. Sample: Collected in Tokyo at the beginning of July, 1932. 1 kg. of large animals was taken. After washing with running water over night, they were dipped into boiling water for 2 minutes and the shell contents were drawn out, on which the sulphates were determined separately as shown in the following scheme. On liquid parts (extracts) both organic and inorganic sulphates were determined, but on the insoluble parts the organic sulphate only was determined, as there might be no insoluble inorganic sulphate remained in the extract residue. Hydrolysis of organic sulphate was conducted by boiling 50 c.c. of extract (or the suspension of extract residue) with 14 c.c. of conc. HCl for 8-12 hrs. under a reflux condenser.

Table I.

Distribution of organic sulphate in some parts of a body of *Viviparus*.

Parts	S mg.	S mg. per 100 gr. sample
Alcohol extract	8.1	—
Muscular part	13.4	9.6
Embryos	23.3	47.6
Organ part	24	12.2
Total	68.8	(mean) 9.8

Scheme of analysis of *Viviparus japonicus*, Martens.



* $S_{(org.)}$ or $S_{(inorg.)}$ means sulphur of organic or inorganic sulphate.

Analyses of 4 Marine Shell-fishes.

Samples: *Haliotis gigantea* Gmelin (Awabi) } from market.
Turbo cornutus Solander (Sazae) }
Charonia lampas Linne (Bōshu-bora) } from Misaki Marine Biological
Strombus luhuanus Linne (Magaki-gai) } Station.

Their shell-contents were boiled for few minutes, ground to paste with some water and sand (free from sulphate) and extracted by 5-12 times of 10% sodium chloride solution in an ice-chamber for 2 days. The liquids were then centrifuged and the sulphates were determined on the supernatant liquid and the precipitate respectively.

Table II.

	Haliotis	*Turbo*	*Charonia*	*Strombus*
Weight gr.	200	131	167	88 (3 shells)
Shell-content gr. (wet)	134	39	37	24
$S_{(org.)}$ mg. in precipitate	8.5	24.0	78.4	29.2
$S_{(org.)}$ mg. in extract	5.4	13.0	17.3	5.3
$S_{(inorg.)}$ mg. in extract	27.4	17.4	27.0	6.7
Per 100 gr. Shell-content $S_{(org.)}$ mg.	10.4	95	259	144
Per 100 gr. Shell-content $S_{(inorg.)}$ mg.	20.5	44.6	73.0	27.9

In comparing these results with the activities of enzyme reported in the previous paper,[1] we see the order of magnitudes of percentage of organic sulphate is in general same as that of activities of enzyme:

Table III.

	Hydrolysing power of enzyme (%)	Org. Sulphate (mg. S)	Total sulphate (mg. S)	Relative weight of shell $\frac{\text{weight of shell}}{\text{Total weight}} \times 100$
		Per 100 gr. shell-content		
Charonia	74.6	259	332	78
Strombus	18.3	144	171.9	73
Haliotis	12.5	10.4	30.9	33
Turbo	7.2	95	139.6	70

Digestion of the Embryos of *Viviparus* by Pepsin. Some embryos of *Viviparus*, after 1 hour's boiling with water, were digested with pancreatin and pepsin-HCl solutions. After 2 days the embryos disappeared in pepsin mixture, giving white turbid solution, which after clarification by Esbach's picric acid reagent and "Silikatzuckerkohle" showed a considerable amount of organic sulphate.

Mucus secreted from *Charonia*. Fresh *Charonia*, when treated with chloroform, secretes a large amount of mucus. The gelatinous mass, formed by the precipitation of mucus with acetone and subsequent washing with water until free from sulphate, gave on hydrolysis a marked amount of sulphate with a smaller amount of phosphate. On the other hand, soluble organic sulphate was found in the mucus, when it was heated to 100° and the heat-coagulum was filtered off. This soluble organic sulphate was precipitated incompletely by lead acetate, but completely by basic lead acetate.

Hydrolysis of Soluble and Insoluble Organic Sulphates by Gluco-sulfatase. The solution of soluble organic sulphate just above mentioned was treated with barium chloride to remove inorganic sulphate and the filtrate was mixed with enzyme obtained from the liver of *Charonia* as follows:

	A	B
Extract of enzyme	10 c.c.	10 c.c.
Filtrate (substrate)	50 c.c.	—
Water + $BaCl_2$	—	50 c.c.
4/10 N-Na acetate	10 c.c.	10 c.c.
Chloroform	1 c.c.	1 c.c.

At the start there was 7.16 mg. S of organic sulphate in A. After standing for a week at 32–33°C., both were centrifuged and the precipitates of barium sulphate formed were separately collected, washed and ignited.

Found: 7.6 mg. $BaSO_4$ in A; 3.7 mg. $BaSO_4$ in B. Therefore 3.9 mg. $BaSO_4$ were produced from substrate by enzyme action.

The action of enzyme upon insoluble organic sulphate was examined as follows: 10 gr. of the insoluble part of *Charonia* (containing 19.1 mg. $S_{(org.)}$, none of inorganic sulphate), prepared in the course of foregoing analysis, were kneaded with 20 c.c. of enzyme solution (aqueous extract of the liver of another *Charonia*, containing none of organic sulphate and 18.8 mg. $S_{(inorg.)}$) and the suspension was kept at 35–36°C. for 2 weeks with some chloroform. At the end of the experiment, there was an increase of inorganic sulphate in liquid portion (11.1 mg. S), that is to say, 58.2% of the insoluble organic sulphate were hydrolysed by the enzyme. Thus, the soluble and insoluble organic sulphates occurring naturally in the shell-fish are attacked by the enzyme.

Thanks are due to Dr. C. Hattori who kindly supplied us the sample of enzyme.

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